

IMMUNIZATION FOR EBOLA VIRUS INFECTION

FIELD OF THE INVENTION

The present invention relates generally to viral vaccines and, more particularly, to Ebola virus vaccines and methods of protecting against disease caused by infection
5 with Ebola virus.

BACKGROUND OF THE INVENTION

The Ebola viruses, and the genetically-related Marburg virus, are filoviruses associated with outbreaks of highly lethal hemorrhagic fever in humans and primates in North America, Europe, and Africa. Peters, C.J. et al., *Filoviridae: Marburg and*
10 *Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Peters, C.J. et al., *Semin. Virol.* 5:147-154 (1994). Ebola viruses are negative-stranded RNA viruses comprised of four subtypes, including those described in the Zaire, Sudan, Reston, and Ivory Coast episodes. Sanchez, A. et al., *PNAS (USA)* 93:3602-3607 (1996). Although several
15 subtypes have been defined, the genetic organization of these viruses is similar, each containing seven linearly arrayed genes. Among the viral proteins, the envelope glycoprotein exists in two alternative forms, a 50-70 kilodalton (kDa) secreted protein of unknown function encoded by the viral genome and a 130 kDa transmembrane glycoprotein generated by RNA editing that mediates viral entry. Peters, C.J. et al.,
20 *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Sanchez, A. et al., *PNAS (USA)* 93:3602-3607 (1996). Other structural gene products include the nucleoprotein (NP), matrix proteins VP24 and VP40, presumed nonstructural proteins VP30 and VP35, and the viral polymerase (reviewed in Peters, C.J. et al., *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996)). Although
25 spontaneous variation of its RNA sequence does occur in nature, there appears to be less nucleotide polymorphism within Ebola subtypes than among other RNA viruses (Sanchez, A. et al., *PNAS (USA)* 93:3602-3607 (1996)), suggesting that immunization
30 may be useful in protecting against this disease. Previous attempts to elicit protective immune responses against Ebola virus using traditional active and passive immunization approaches have, however, not succeeded. Peters, C.J. et al., *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Clegg, J.C.S.

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et al., *New Generation Vaccines*. (eds., Levine, M.M., Woodrow, G.C., Kaper, J.B. & Cobon, G.S.) 749-765 (New York, NY, Marcel Dekker, Inc. 1997); Jahrling, P.B. et al., *Arch. Virol. Suppl.* 11:135-140 (1996).

It would thus be desirable to provide a vaccine to protect against disease
5 caused by infection with Ebola virus. It would further be desirable to provide methods of making and using said vaccine.

SUMMARY OF THE INVENTION

Ebola virus vaccines comprising nucleic acid molecules encoding Ebola viral proteins are provided. In one embodiment, the nucleic acid molecule encodes the
10 transmembrane form of the viral glycoprotein (GP). In another embodiment, the nucleic acid molecule encodes the secreted form of the viral glycoprotein (sGP). In yet another embodiment, the nucleic acid molecule encodes the viral nucleoprotein (NP).

The present invention also provides methods for immunizing a subject against
15 disease caused by infection with Ebola virus comprising administering to the subject an immunoeffective amount of an Ebola virus vaccine. Administration can be by any of the routes normally used for gene therapy. In a preferred method, the Ebola virus vaccine is administered by intramuscular injection. The genetic immunization methods of the present invention provide protective immunity against disease caused
20 by infection with Ebola virus.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

25 The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings.

Figures 1A and 1B are photographs showing expression of Ebola virus gene products in eukaryotic plasmid expression vectors.

30 *Figure 1A.* Expression vectors encoding the indicated viral gene products under regulation of the CMV immediate-early region 1 enhancer and promoter were prepared and transfected into 293 cells as previously described. Manthorpe, M. et al. *Hum. Gene. Ther.* 4:419-431 (1993); Sambrook, J., Fritsch, E.F., & Maniatis, T. Cold Spring Harbor, N.Y. Cold Spring Laboratory Harbor Press, 1994. Cell extracts
35 were prepared and analyzed by Western blot analysis for NP (left) or GP (right) using

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relevant rabbit antisera and a secondary antibody, horseradish peroxidase conjugated donkey anti-rabbit IgG of a dilution of 1:5,000. Incubation with primary antibody was for 30 minutes at room temperature, and for 30 minutes at room temperature with secondary antibody. Immunocomplexes were then detected by chemiluminescence using super signal substrate reagents (Pierce) according to manufacturer's instructions.

Figure 1B. Generation of antibody response in mice immunized with the indicated vectors and analyzed by Western blot for NP, GP, and sGP as shown. Antisera from mice were tested at a dilution of 1:500 (NP), 1:50 (GP), or 1:50 (sGP), respectively, and developed with a secondary antibody (sheep anti-mouse, 1:5,000, Amersham Life Science) and chemiluminescence as in Figure 1A. The control vector used for immunization represents the expression vector plasmid with no insert. Manthorpe, M. et al., *Hum. Gene. Ther.* 4:419-431 (1993).

Figures 2A-2D are graphs showing the immune responses to NP and GP after genetic immunization in mice.

Figure 2A. Splenic lymphocytes from vector or NP-plasmid immunized mice were isolated approximately 6 weeks after the initial immunization and sensitized *in vitro* for 5 days with 10 U/ml hIL-2. Renca-NP cells sensitized splenocytes from vector-immunized or pCMV-NP immunized mice were used to detect CTL activity at the indicated effector:target ratios on Renca or Renca-NP cells (left, middle) or with allogeneic effector cells with Renca-NP to show that they are susceptible to lysis (right). Allogeneic effector cells were generated by incubating cells derived from mice with a C57Bl/6 background (5×10^6 /ml) with irradiated Balb/c spleen cells (5×10^6 /ml) in the presence of IL-2 (20 U/ml) for five days. The chromium release CTL assay with Renca-NP cells was performed in triplicate as previously described. Ohno, T. et al., *Gene. Ther.* 4:361-366 (1997).

Figure 2B. Balb/C female mice were immunized with the sGP plasmid expression vector and analyzed for their ability to lyse the syngeneic Renca cell line stably expressing GP. Isolation of stable transfectants, confirmation of expression, and CTL assay were performed as described (see, Specific Example, II. Methods). Renca-GP or sGP sensitized splenocytes from pCMV-GP or pCMV-sGP immunized mice were used to determine the specific killing of 51 chromium labeled Renca-GP cells at the indicated E/T ratios.

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Figure 2C. Mice immunized with GP were analyzed for their ability to lyse a syngeneic CT26 cell stably expressing GP or CT26 vector control transduced line at the indicated E/T ratios.

Figure 2D. Cellular proliferative response in the indicated immunized mice. T cells, enriched or depleted (see, Specific Example, II. Methods), were incubated at 10^5 cells/ml with sGP condition media (25%). Background was determined with cells incubated in media from control transfected 293 cells and subtracted from proliferation seen in sGP-containing supernatants.

Figures 3A-3C are graphs showing immunization with sGP or GP expression plasmids induces T cell responses to sGP in guinea pigs.

Figures 3A-3C. Cell-mediated immunity in guinea pigs was analyzed by performing assays to detect cell proliferation to control or GP antigen (A) or T-cell growth factor production in response to the indicated antigens. The culture supernatants containing these antigens were prepared as previously described (Bottomly, K. et al., Measurement of human and murine interleukin 2 and interleukin 4. in *Current Protocols in Immunology*. (eds., Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. & Strober, W.) 6.3.1-6.3.12 (New York, John Wiley & Sons, Inc. 1992); Arai, H. et al., *Nat. Med.* 3:843-848 (1997)), and included at a final concentration of 10% (volume/volume). In A, cell numbers refer to the concentration of spleen cells per ml in the ^3H -thymidine proliferation assay. In B, supernatants from A, harvested at the time of the peak proliferative response to sGP, were incubated with primary guinea pig T cells maintained in 200 U/ml of human IL-2. The percent maximal response refers to the magnitude of stimulation in response to the indicated stimuli relative to supernatants from 24 hour concanaval (in A-stimulated cells (2 $\mu\text{g/ml}$)). The requirement of T lymphocytes in guinea pig spleen cells for the proliferative response to sGP, performed as described in Specific Example, II. Methods, is shown (C).

Figures 4A-4F are photographs showing the immunohistochemical analysis of Ebola virus antigens in liver, lung, and spleen from representative protected (GP-animal 3) or infected (vector-animal 2) guinea pigs.

Figures 4A-4F. Magnification: liver, 40x; lung, 20x; spleen, 20x.

Figure 5 is a schematic of the plasmid pVR 1012-GP(IC) (Ivory Coast strain of GP, SEQ ID NO: 1).

Figure 6 is a schematic of the plasmid pVR 1012-GP(S) (Sudan strain of GP, see SEQ ID NO: 2).

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Figure 7 is a schematic of the plasmid pVR 1012-GP(Z) (Zaire strain of GP, see SEQ ID NO: 3).

Figure 8 is a schematic of the plasmid pVR 1012-sGP(Z) (Zaire strain of sGP, see SEQ ID NO: 4).

5 Figure 9 is a schematic of the plasmid pVR 1012-NP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Ebola virus vaccines are provided comprising a nucleic acid molecule encoding an Ebola viral protein operatively-linked to a control sequence in a pharmaceutically acceptable carrier. In one embodiment, the nucleic acid molecule encodes the
10 transmembrane form of the viral glycoprotein (GP). In another embodiment, the nucleic acid molecule encodes the secreted form of the viral glycoprotein (sGP). In yet another embodiment, the nucleic acid molecule encodes the viral nucleoprotein (NP).

The present invention further includes vaccines comprising nucleic acid
15 molecules encoding Ebola viral proteins other than GP, sGP, and NP, e.g., other structural gene products which elicit protective immunity from disease caused by infection with Ebola virus. The nucleic acid molecules of the vaccines of the present invention encode structural gene products of any Ebola viral strain including the Zaire, Sudan, Ivory Coast and Reston strains. Nucleic acid molecules encoding structural
20 gene products of the genetically-related Marburg virus strains may also be employed. Moreover, the nucleic acid molecules of the present invention may be modified, e.g., the nucleic acid molecules set forth herein may be mutated, as long as the modified expressed protein elicits protective immunity from disease caused by infection with Ebola virus. For example, the nucleic acid molecule may be mutated so that the
25 expressed protein is less toxic to cells. The present invention also includes vaccines comprising a combination of nucleic acid molecules. For example, and without limitation, nucleic acid molecules encoding GP, sGP and NP of the Zaire, Sudan and Ivory Coast Ebola strains may be combined in any combination, in one vaccine composition.

30 The present invention also provides methods for immunizing a subject against disease caused by infection with Ebola virus comprising administering to the subject an immunoeffective amount of an Ebola virus vaccine. Methods of making and using Ebola virus vaccines are also provided by the present invention including the preparation of pharmaceutical compositions.

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As referred to herein, the term "encoding" is intended to mean that the subject nucleic acid may be transcribed in a cell, e.g., when the subject nucleic acid is linked to appropriate control sequences such as a promoter in a suitable vector (e.g., an expression vector) and the vector is introduced into a cell. The nucleic acid molecules of the present invention may be DNA molecules, cDNA molecules or RNA molecules, and are preferably cDNA molecules. The term "operatively-linked" as used herein refers to functional linkage between a nucleic acid expression control sequence (such as a promoter) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence. Expression control sequences are known to those skilled in the art (see, e.g., Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990)). Vectors which contain both a promoter and a cloning site to which an inserted piece of nucleic acid is operatively-linked to the promoter, are well known in the art and are generally referred to herein as "expression vectors" or "expression vector plasmids". Preferably, these vectors are capable of transcribing nucleic acid *in vitro* and *in vivo*. A preferred vector is the cytomegalovirus (CMV) expression vector which directs high levels of gene expression in muscle.

Nucleic acid molecules which hybridize under stringent conditions to the nucleic acid molecules described herein are also within the scope of the present invention. As will be appreciated by those skilled in the art, multiple factors are considered in determining the stringency of hybridization including species of nucleic acid, length of nucleic acid probe, T_m (melting temperature), temperature of hybridization and washes, salt concentration in the hybridization and wash buffers, aqueous or formamide hybridization buffer, and length of time for hybridization and for washes. An example of stringent conditions are DNA-DNA hybridization with a probe greater than 200 nucleotides in 5 x SSC, at 65°C in aqueous solution or 42°C in formamide, followed by washing with 0.1 x SSC, at 65°C in aqueous solution. (Other experimental conditions for controlling stringency are described in Maniatis, T. et al., *Molecular Cloning: a Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Springs, N.Y. (1982) at pages 387-389 and Sambrook, J. et al., *Molecular Cloning: a Laboratory Manual*, Second Edition, Volume 2, Cold Springs Harbor Laboratory, Cold Springs, N.Y., at pages 8.46-8.47 (1989)).

It will be appreciated that administration of the vaccines of the present invention can be by any of the routes normally used for gene therapy. In a preferred

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method, administration is by intramuscular injection, however, other procedures for transfecting cells may also be employed, such as transfection using calcium phosphate coprecipitation, liposome-mediated transfection, plasmid and viral vector-mediated transfection and DNA protein complex-mediated transfection. Viral vector-mediated transfection includes, without limitation, the use of retroviral, replication-deficient retroviral, adenoviral and adeno-associated viral vectors. Cells transfected by the vaccines in the context of *ex vivo* gene therapy can also be administered.

It will be appreciated that more than one route of administering the vaccines of the present invention may be employed either simultaneously or sequentially (*e.g.*, boosting). In addition, the vaccines of the present invention may be employed in combination with traditional immunization approaches such as employing protein antigens, vaccinia virus and inactivated virus, as vaccines. Thus, in one embodiment, the vaccines of the present invention are administered to a subject (the subject is "primed" with a vaccine of the present invention) and then a traditional vaccine is administered (the subject is "boosted" with a traditional vaccine). In another embodiment, a traditional vaccine is first administered to the subject followed by administration of a vaccine of the present invention. In yet another embodiment, a traditional vaccine and a vaccine of the present invention are co-administered.

Immunogenicity may be significantly improved if the vaccines of the present invention are co-administered with an immunostimulatory agent or adjuvant. Adjuvants enhance immunogenicity but are not necessarily immunogenic themselves. Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Adjuvants may thus be employed to enhance the immunogenicity of the vaccines of the present invention, as well as the immunogenicity of traditional vaccines. Suitable adjuvants are well known to those skilled in the art and include, without limitation, aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, a lipoprotein, ISCOM matrix, DC-Chol, DDA, and other adjuvants and bacterial toxins, components and derivatives thereof.

The vaccines of the present invention may also be co-administered with cytokines to further enhance immunogenicity. The cytokines may be administered by methods known to those skilled in the art, *e.g.*, as a nucleic acid molecule in plasmid form or as a protein or fusion protein.

Upon inoculation with a pharmaceutical composition as described herein, the immune system of the host responds to the vaccine by producing antibodies, both secretory and serum, specific for Ebola virus proteins. As a result of the vaccination, the host becomes at least partially or completely immune to Ebola virus infection, or
5 resistant to developing moderate or severe disease caused by Ebola virus infection. Although Ebola virus infection and disease caused thereby are discussed in detail herein, it will be appreciated that the vaccines and methods of the present invention may be employed to immunize a subject against hemorrhagic fever generally, such as that caused by infection by the genetically-related Marburg virus.

10 Pharmaceutical compositions comprising the nucleic acid molecules encoding Ebola viral proteins described herein, either alone or in combination, and a pharmaceutically acceptable carrier, are also provided by the present invention. As used herein, the phrase "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as those suitable for parenteral
15 administration, such as, for example, by intramuscular, intraarticular (in the joints), intravenous, intradermal, intraperitoneal, and subcutaneous routes. Examples of such formulations include aqueous and non-aqueous, isotonic sterile injection solutions, which contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-
20 aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the vaccine dissolved in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined
25 amount of the vaccine, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; (d) suitable emulsions; and (e) polysaccharide polymers such as chitians. The vaccine, alone or in combination with other suitable components, may also be made into aerosol formulations to be administered via inhalation, e.g., to the bronchial passageways. Aerosol formulations can be placed into pressurized
30 acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the vaccine with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons.
35 In addition, it is also possible to use gelatin rectal capsules which consist of a

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combination of the vaccine with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the recipient, e.g., the patient.

5 The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules or vials and may be prepared by any method known in the art.

Pharmaceutical compositions comprising any of the nucleic acid molecules encoding Ebola viral proteins of the present invention are useful to immunize a subject against disease caused by Ebola virus infection. Thus, this invention further
10 provides methods of immunizing a subject against disease caused by Ebola virus infection, e.g., hemorrhagic fever, comprising administering to the subject an immunoeffective amount of a pharmaceutical composition of the invention. This subject may be an animal, for example a mammal, such as a primate or preferably a human.

15 The vaccines of the present invention are also suitable for veterinary immunization. The vaccines of the present invention comprising nucleic acid molecules encoding Ebola virus structural gene products from the Reston strain, which is known to infect animals, are particularly useful in such veterinary immunization methods.

20 The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic and protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the immune system of the individual to synthesize antibodies, and, if needed, to produce a cell-mediated immune response.
25 Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and may be monitored on a patient-by-patient basis. However, suitable dosage ranges are readily determinable by one skilled in the art and generally range from about 300 μ g to about 4-5 mg. The dosage may also depend, without limitation, on the route of administration, the patient's state of health
30 and weight, and the nature of the formulation.

Methods of immunizing a subject against multiple strains of Ebola virus are further provided herein. The nucleic acid molecules encoding Ebola viral proteins of the present invention may be combined with nucleic acid molecules encoding other viral proteins of other virus strains to achieve protection against multiple strains of

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Ebola virus. Typically the vaccines will be in an admixture and administered simultaneously, but may also be administered separately.

In some instances it may be desirable to combine the Ebola virus vaccines of the present invention with vaccines which induce protective responses to other agents, particularly other viruses. For example, the vaccine compositions of the present invention can be administered simultaneously, separately or sequentially with other genetic immunization vaccines such as those for influenza (Ulmer, J.B. et al., *Science* 259:1745-1749 (1993); Raz, E. et al., *PNAS (USA)* 91:9519-9523 (1994)), malaria (Doolan, D.L. et al., *J. Exp. Med.* 183:1739-1746 (1996); Sedegah, M. et al., *PNAS (USA)* 91:9866-9870 (1994)), and tuberculosis (Tascon, R.C. et al., *Nat. Med.* 2:888-892 (1996)).

It will also be appreciated that single or multiple administrations of the vaccine compositions of the present invention may be carried out. For example, subjects who are particularly susceptible to Ebola virus infection may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

This invention also provides kits comprising the vaccines of the present invention. For example, kits comprising a vaccine and instructions for use are within the scope of this invention.

The vaccines and methods of the present invention evoke a protective immune response and do not lead to immunopotential or exacerbated disease. The vaccines lack transmissibility, are genetically stable and induce protective levels of humoral and cell-mediated immunity.

In order to more fully demonstrate the advantages arising from the present invention, the following example is set forth. It is to be understood that the following is by way of example only and is not intended as a limitation on the scope of the invention.

SPECIFIC EXAMPLE

I. RESULTS

Immune response to viral gene products in mice. To characterize immune responses to selected Ebola virus proteins, eukaryotic expression vector plasmids were injected into mice. The cytomegalovirus (CMV) immediate early region 1 enhancer was used to stimulate transcription because it directs high levels of gene

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expression in muscle. Manthorpe, M. et al., *Hum. Gene. Ther.* 4:419-431 (1993). cDNAs encoding an abundant structural protein, the major viral nucleocapsid phosphoprotein (NP), the secreted glycoprotein (sGP), or the membrane-associated glycoprotein (GP) were inserted. Alternative forms of GP were chosen because it had

5 been postulated that the transmembrane protein contained a protein sequence motif also found in oncogenic retroviruses that might suppress immune responses. Burkreyev, A.A. et al., *FEBS. Lett.* 323:183-187 (1993); Cianciolo, G.J. et al., *Science* 230:453-455 (1985); Harris, D.T. et al., *J. Immunol.* 138:889-894 (1987); Volchkov, V.E. et al., *FEBS. Lett.* 305:181-184 (1992); Sanchez, A. et al., *Virus. Res.* 29:215-

10 240 (1993). Expression of the relevant proteins was confirmed after transfection of the human renal epithelial cell line, 293, by immunoblotting with antisera to these gene products (Fig. 1A). For NP, the expected full-length 104 kDa protein normally produced by the virus was seen, together with lower molecular weight species likely generated from truncated protein or degradation products described previously.

15 Sanchez, A. et al., *Virology* 170:81-91 (1989). Similarly, expression of sGP and GP revealed a heterogeneous pattern whose sizes correlated with the expected products of cleavage or post-translational carbohydrate modification. Sanchez, A. et al., *PNAS (USA)* 93:3602-3607 (1996).

These plasmids were injected into mice to characterize their ability to induce

20 humoral and cellular immune responses to the relevant viral proteins. Three injections, each with 50 μ g of plasmid DNA in saline (100 μ l), were performed at two-week intervals in Balb/C female mice (6-8 week old, Charles River). Serum from immunized recipients were examined for antibody responses. An antibody response to the viral NP gene product was readily detectable (Fig. 1B), with titers of \geq

25 1:16,000 by Western blot analysis. The titer of antibody induced in response to injection with plasmids encoding the viral glycoproteins was lower. After immunization with GP, no antibody was detectable by Western blotting, while immunization with sGP induced an antibody response (Fig. 1B). The more sensitive ELISA (Ksiazek, T.G., *Lab. Anim.* 20:34-46 (1991); Ksiazek, T.G. et al., *J. Clin. Microbiol.* 30:947-950

30 (1992)) did allow detection of antibodies to both GP and sGP at titers of 1:400 and 1:1,200, respectively. Cytolytic T cell (CTL) responses to these viral proteins were analyzed next. Despite the substantial humoral immune response to NP, minimal CTL activity was detected against syngeneic cells expressing this viral protein (Fig. 2A). In contrast, genetic immunization with sGP, which elicited a weaker antibody

35 response, induced a marked cytolytic T cell response to cells expressing GP (Fig. 2B).

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Immunization with the GP plasmid also induced a significant CTL response to GP (Fig. 2C). These data suggested that both the secreted and transmembrane form of the protein could be processed for antigen presentation and the transmembrane form was a target for recognition by these cytolytic T cells. Finally, antigen-specific T cell proliferation to sGP was also observed in GP and sGP but not plasmid control injected mice (Fig. 2D).

The ability of antibodies detected in mouse sera after immunization to neutralize virus was tested in an *in vitro* infection assay. McCormick, J.B. et al., *J. Infect. Dis.* 147:264-267 (1983). In no case was neutralization of infectivity observed, even at titers of 1:10 (data not shown), despite the documented presence of antibody after NP and sGP immunization by Western blot analysis. Infectivity *in vitro* was thus not inhibited by Ebola-specific antibodies.

Immune function and viral challenge in guinea pigs. To determine whether the *in vivo* immune responses could protect against viral infection, virus was adapted to grow in guinea pigs. Though this species is not well-suited to analysis of immune function, infection in adult mice has not been successful. Moreover, infection in guinea pigs, used originally to propagate virus from infected humans, is a well-established animal model for the human disease. Infection gives rise to a syndrome of hemorrhagic fever with levels of virus, organ pathology, and infection of reticuloendothelial and mononuclear cells comparable to humans. Bowen, E.T.W. et al., *Lancet* 1:571-573 (1977).

Two groups of immunized guinea pigs were studied. Animals were injected intramuscularly with the relevant expression vector plasmids, and the response to infection in groups immunized with either sGP, GP, NP, or control plasmids was observed. In the first group, animals were challenged within 2 months after the initial immunization, at which time the antibody titers were high, ranging from 1:1,600 to >1:25,000 (Table 1A). In these animals, nearly complete protection from lethal challenge was observed in GP (6/6), sGP (5/6), and NP (4/4) immunized subjects, in contrast to controls (0/6). In a second group, guinea pigs were challenged four months after the initial immunization (Table 1B). As in the first group, all animals immunized with the control vector succumbed to infection within a week after virus challenge (n=4). In this group, antibody titers were lower, and three of the four guinea pigs immunized with NP succumbed to infection, with the single survivor appearing severely ill after 1 week, in contrast to the protective response with NP at the earlier time point after immunization in Group I. More effective protection was

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seen in animals immunized with vector expressing GP, protection was noted in four of five animals challenged, with one survivor appearing weak but surviving the viral challenge. Similarly, three of the five animals immunized with sGP showed no ill effects following viral challenge. Protection in this group again correlated with the ability to sustain an effective immune response to GP or sGP. Together, all guinea pigs immunized with vectors expressing GP or sGP which had titers greater than 1:5,120 were resistant to infection (11/11) compared to 0/10 controls ($p=0$, by Fisher's exact test). Twelve of fourteen animals with antibody titers $\geq 2,560$ survived viral challenge compared to controls ($p=.00003$, by Fisher's exact test). Similar to immunized mice, guinea pigs injected with GP or sGP plasmids were able to generate cell-mediated immune responses to the viral glycoprotein in addition to the antibody response. These responses were antigen-specific and T cell-dependent, as detected in sGP antigen-dependent spleen cell proliferation and T-cell growth factor assays (Fig. 3A-C). Thus, the ability to generate and sustain significant cellular immune responses *in vivo* correlated with protection from infection. Though antibody titer correlated with protection, cell-mediated immunity appeared necessary for protection since passive transfer of antibody to GP does not confer protection (Peters, C.J. et al., *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Jahrling, P.B. et al., *Arch. Virol. Suppl.* 11:135-140 (1996)) and antisera from protected guinea pigs did not inhibit virus replication *in vivo* ($n=3$) or at a 1:10 dilution *in vitro* (data not shown). Since the Hartley guinea pig to which the virus has been adapted for growth is outbred, cellular adoptive transfer studies could not be performed.

TABLE 1 - Group I

	<u>Plasmid</u>	<u>Subject</u>	<u>ELISA(Pre)</u>	<u>ELISA(Post)</u>	<u>Viral Ag</u>	<u>Survival</u>
	GP	1	>1:25,600	1:12,800	-	Yes
	GP	2	>1:25,600	1:25,600	-	Yes
	GP	3	>1:25,600	1:25,600	-	Yes
30	GP	4	1:25,600	1:6,400	-	Yes
	GP	5	1:25,600	1:12,800	-	Yes
	GP	6	1:25,600	1:25,600	-	Yes
	SGP	1	1:12,800	1:25,600	-	Yes
	SGP	2	1:6,400	1:25,600	-	Yes
35	SGP	3	1:6,400	1:25,600	-	Yes

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5	SGP	4	1:25,600	1:25,600	-	Yes
	SGP	5	>1:25,600	1:12,800	-	Yes
	SGP	6	1:1,600	Negative	+	No
	NP	1	1:12,800	>1:25,600	-	Yes
	NP	2	>1:25,600	1:25,600	-	Yes
10	NP	3	1:12,800	1:12,800	-	Yes
	NP	4	1:25,600	1:25,600	-	Yes
	Vector alone	1	Negative	Negative	+	No
	Vector alone	2	Negative	n.d.	+	No
	Vector alone	3	Negative	Negative	+	No
	Vector alone	4	Negative	Negative	+	No
	Vector alone	5	Negative	n.d.	+	No
	Vector alone	6	Negative	n.d.	+	No

Guinea pigs were immunized on days 1, 14, 28, 42, and challenged on day 62.

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TABLE 1 - Group II

	<u>Plasmid</u>	<u>Subject</u>	<u>ELISA(Pre)</u>	<u>ELISA(Post)</u>	<u>Viral Ag</u>	<u>Survival</u>
20	GP	1	1:2,560	n.d.	+/-	No
	GP	2	1:5,120	1:10,240	-	Yes
	GP	3	1:10,240	1:10,240	-	Yes
	GP	4	1:1,280	n.d.	+/-	No
	GP	5	1:5,120	1:20,480	-	Yes (ill)
25	SGP	1	1:2,560	n.d.	+	No
	SGP	2	1:10,240	1:5,120	+/-	Yes
	SGP	3	1:10,240	1:81,920	-	Yes
	SGP	4	1:2,560	1:5,120	-	Yes
	SGP	5	1:640	n.d.	+	No
30	NP	1	n.d.	n.d.	+	No
	NP	2	n.d.	n.d.	+	No
	NP	3	n.d.	n.d.	+	No
	NP	4	n.d.	Negative	+	Yes (ill)
	Vector alone	1	Negative	n.d.	+	No
	Vector alone	2	Negative	n.d.	+	No
	Vector alone	3	Negative	n.d.	+	No
	Vector alone	4	Negative	n.d.	+	No

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Guinea pigs were immunized on days 1, 14, 42, and 112 and challenged on day 122.

n.d.=not done. Viral ag denotes presence of virus determined by immunohistochemistry (30) performed on spleen, liver, lung, kidney, and heart tissues; "+" = widespread systemic involvement of the mononuclear phagocyte system and to a lesser extent endothelial and parenchymal cells; "+/f" = focal involvement (seen in the spleen of SGP #2, the liver and spleen of GP #1, and the lung of GP#4) where rare sites of anti-Ebola antibody staining were detected.; "-" = no Ebola virus antigen detected in tissues.

ELISA determinations made prior to viral challenge (Pre) or at least 7 days after (Post) infection, respectively.

The surviving NP immunized animal (4) was found to have significant levels of virus in major organs by immunohistochemistry, and more than 5 logs of virus was detected in the serum and spleen, in contrast to GP and sGP animals where no virus was detected.

Histopathologic analysis of infection in immunized guinea pigs.

Pathologic analysis revealed widespread tissue necrosis and dissemination of virus by immunohistochemistry, similar to human disease. Virus load correlated with susceptibility to infection with titers of $\geq 10^5$ in infected animals compared to undetectable levels in immunized survivors. In infected animals, the liver, lung, and spleen showed evidence of significant viral antigen by immunohistochemistry (Fig. 4, Table 1), and both reticuloendothelial and mononuclear phagocytic involvement was observed.

Determination of antibody response in animals which survived virus challenge revealed increases in the immune response to viral proteins when initial titers were lower (Table 1). Less consistent increases in antibody titers were observed in the NP immunized animals. These data suggest that Ebola virus infection may stimulate immunity in survivors of a viral challenge when immune responses are not optimal.

II. METHODS

Plasmids. Plasmids containing the GP, sGP, or NP cDNAs (Sanchez, A. et al., *Virus. Res.* 29:215-240 (1993), Genbank) were used to subclone the relevant inserts into CMV expression vectors which utilized the bovine growth hormone polyadenylation sequence. Manthorpe, M. et al., *Hum. Gene. Ther.* 4:419-431 (1993). (see Figures 5-9 and SEQ ID NOS: 1-4). Briefly, for GP, plasmid pGEM-3Zf(-)-GP was digested with EcoR I, treated with the Klenow fragment of E. coli DNA polymerase, and digested with BamH I. The GP fragment was then inserted into the pCMV expression vector plasmid digested with BamH I, Klenow fragment and Bgl II. For sGP, the plasmid pCRII-sGP was digested with EcoR I, treated with Klenow

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enzyme, and the resulting fragment inserted into the BamH I/Bgl II CMV plasmid which had been incubated with Klenow fragment, calf intestinal phosphatase (CIP), then phenol chloroform extract. For the NP expression vector, plasmid pSP64-NP2 (Sanchez, A. et al., *Virology* 170:81-91 (1989)) was digested with EcoR I, treated with
5 Klenow enzyme, and digested with BamH I. The NP insert was cloned into CMV treated with BamH I, Klenow enzyme, followed by heat inactivation and Bgl II digestion.

Cell lines and transfectants. For stable transfectants, the relevant cDNAs were inserted into a CMV expression plasmid containing a neomycin resistant gene, pCMV-neo (H. Arai, unpublished data), which was digested with Xba I, and treated
10 with CIP and Klenow enzyme. The EcoR I/BamH I GP fragment from pGEM-3Zf(-)-GP, the EcoR I sGP fragment from pCRII-SGP, or the EcoR I/BamH I NP fragment from pSP64-NP2 was treated with Klenow enzyme and ligated to this plasmid backbone. These vectors were transfected into Renca or CT26 which was syngeneic
15 to Balb/C mice using calcium phosphate and selected in 0.7 or 1mg/ml G418 for 2-6 weeks. Expression of GP, sGP, or NP from these vectors in Renca or CT26 cells was also confirmed by Western blot analysis (data not shown).

Cell proliferation assay. Spleen cells from male Hartley guinea pigs or Balb/C female mice (8-10 weeks) immunized with the indicated plasmid expression
20 vectors were incubated with sGP or vector control supernatants (25% volume:volume) from transfected 293 cells at the indicated cell concentrations. T cell depletion was performed using the CT5 monoclonal antibody (Tan, B.T.G. et al., *Hybridoma* 4:115-124 (1985)) (Biosource, Camarillo, CA) for guinea pigs or anti-Thy 1.2 antibody in the mouse using immunomagnetic microbeads (Miltenyi Biotec, Inc., Auburn, CA).

Viral challenge in guinea pigs. Animals were immunized by injection of 100
25 μ l (0.5 mg/ml) in each hind leg (two injections at each time point) with the indicated plasmid expression vectors. Animals were challenged by inoculation with a stock of Ebola virus (Zaire, 1976) that had been passaged once in vero E6 cells and serially passaged by intraperitoneal injection of spleen homogenates in Hartley guinea pigs
30 seven times. Immunized guinea pigs were injected intraperitoneally with 0.5 ml of a 1:1,000 dilution of spleen cell homogenate in Hank's balanced salt solution 122 days after the initial plasmid DNA injection (1000 pfu). Survival was determined 10 days later at which times animals were sacrificed for serologic and pathologic analysis. ELISA, enzyme-linked immunosorbent assay (Volchkov, V.E. et al., *FEBS. Lett.*
35 305:181-184 (1992); Sanchez, A. et al., *Virus. Res.* 29:215-240 (1993)) on infected

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cell supernatants and enriched viral extracts containing GP, sGP, or NP were performed as previously described.

III. DISCUSSION

Following the initial report that injection of plasmid DNA into muscle could
5 direct the synthesis of recombinant proteins (Wolff, J.A. et al., *Science* 247:1465-1468 (1990)), the suggestion was made that this gene transfer approach may be useful for vaccination and was termed genetic immunization. Tang, D.C. et al., *Nature* 356:152-154 (1992). This approach has been applied to different infectious diseases, including influenza (Ulmer, J.B. et al., *Science* 259:1745-1749 (1993); Raz, E. et al., *PNAS* 10 (USA) 91:9519-9523 (1994)), malaria (Doolan, D.L. et al., *J. Exp. Med.* 183:1739-1746 (1996); Sedegah, M. et al., *PNAS* (USA) 91:9866-9870 (1994)), and tuberculosis (Tascon, R.C. et al., *Nat. Med.* 2:888-892 (1996)) and has also been used to modulate antibody and cell-mediated immune responses in autoimmune and allergic diseases. Raz, E. et al., *PNAS* (USA) 90:4523-4527 (1993); Waisman, A. et al., *Nat.* 15 *Med.* 2:899-905 (1996); McCormick, J.B. et al., *J. Infect. Dis.* 147:264-267 (1983); Border, W.A. et al., *Nat. Med.* 1:1000-1001 (1995).

The immune response to selected Ebola virus proteins after genetic immunization in mice was analyzed and their ability to protect against lethal infection in a susceptible animal model, the guinea pig, was tested. The immune analyses
20 performed in different species suggest similar patterns of response, though the specific peptides which may be recognized by the immune system to confer protection in the guinea pig could differ from the mouse. Because the principles of MHC antigen presentation and recognition apply broadly across species (Monaco, J.J., *Immunol. Today* 13:173-179 (1992); Jorgensen, J.L. et al., *Annu. Rev. Immunol.* 10:835-873 25 (1992); Zinkernagel, R.M. et al., *Immunol. Today* 18:14-17 (1997)), the finding that protection was observed in different members of an outbred strain and that similar immune responses were seen in different species is not unexpected and suggests that this approach may be applicable to humans.

Immunization with plasmids encoding distinct viral proteins induced different
30 antibody and cytolytic T cell responses. The broadest immune response was conferred by GP and sGP, which induced both cellular and humoral immunity to the membrane-associated GP. In guinea pigs challenged with doses of virus that are otherwise lethal, sGP provided nearly equivalent protection to GP, with no significant difference between these groups. The ability of vectors expressing GP to confer
35 immunity may be explained by the generation of lower molecular weight degradation

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products (Fig. 1B) which could provide sufficient protein for antigen presentation to induce detectable, cellular, and humoral immune responses in guinea pigs.

Despite the fact that plasmid DNA injection has been shown to affect the immune response to different antigens in infectious and autoimmune diseases, the ability of individual gene products to protect against disease *in vivo* is not readily predictable. In particular, the rapid rates of Ebola virus replication and the poor immunogenicity of its proteins had previously rendered it resistant to immune interventions. Several attempts to confer protection with passive transfer of immunoglobulin were unsuccessful (Peters, C.J. et al., *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Jahrling, P.B. et al., *Arch. Virol. Suppl.* 11:135-140 (1996)), in agreement with the finding set forth herein that antisera from protected animals fails to neutralize virus replication *in vitro*. Previous studies using formalin-fixed virus or purified viral proteins for immunization have also not proven effective.

Peters, C.J. et al., *Filoviridae: Marburg and Ebola Viruses*. in *Fields Virology*. (eds., Fields, B.N., Knipe, D.M. & Howley, P.M.) 1161-1176 (Philadelphia, Lippincott-Raven, 1996); Clegg, J.C.S. & Sanchez, A. Vaccines against arenaviruses and filoviruses. in *New Generation Vaccines*. (eds., Levine, M.M., Woodrow, G.C., Kaper, J.B. & Cobon, G.S.) 749-765 (New York, NY, Marcel Dekker, Inc. 1997).

It is likely that traditional immunization approaches using protein antigens, vaccinia virus, or inactivated virus do not allow for appropriate uptake and presentation of viral antigens by dendritic or other antigen-presenting cells to induce protective immune responses. It has been shown recently that genetic immunization leads to production of recombinant protein(s) in muscle which are delivered to bone marrow-derived antigen-presenting cells. Iwasaki, A. et al., *J. Immunol.* 159:11-14 (1997); Doe, B. et al., *PNAS (USA)* 93:8578-8583 (1996); Corr, M. et al., *J. Exp. Med.* 184:1555-1560 (1996). Synthesis of Ebola glycoprotein after gene transfer apparently allows more efficient processing and presentation and the generation of immune responses not seen with virus or with viral vectors. GP is a large molecule which contains both T and B cell epitopes. Although antibody levels provide a surrogate marker of protection, the fact that passive transfer of antibody did not confer protection implies that immunoglobulin switching and synthesis is reflective of the T helper response to GP. Genetic immunization stimulates T helper cells to generate both CTL and B cell antibody responses to the virus. Although antibody production confirms effective immunization, a productive T cell response, likely involving T_H1 cell

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stimulation, as shown by the T cell proliferation and CTL assays (Fig. 3), is needed for effective immunity. Taken together, these studies suggest that transcription and translation of viral genes in host cells by genetic immunization induces alternative, more effective, processing and antigen presentation which better stimulates immunity to Ebola virus. Since there are yet no effective antiviral agents, the ability to generate protective immunity by vaccination may prove useful in selected high risk populations, particularly in regions of ongoing outbreaks, and among medical and laboratory personnel exposed to the virus. Although it remains important to identify agents which treat acute infection, genetic immunization may help to limit the spread of this highly lethal infectious disease.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

All references cited herein are incorporated by reference as if fully set forth.